

Molecular Cloning of GA 2-Oxidase3 from Spinach and Its Ectopic Expression in *Nicotiana sylvestris*¹

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Previous work has shown that 13-hydroxylated gibberellins (GAs) are predominant in the long-day (LD) plant spinach (*Spinacia oleracea*; GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₁, GA₈, and GA₂₉). Also present in spinach are 2 β -hydroxylated C₂₀-GAs: GA₉₇, GA₉₈, GA₉₉, and GA₁₁₀. Levels of the most abundant GA, GA₉₇, decreased when plants were transferred from short photoperiods (SD) to LD. When [¹⁴C]GA₅₃ was fed to spinach plants, more GA₅₃ was converted to GA₉₇ in SD than in LD, and more radioactive GA₂₀ was formed in LD than in SD. SoGA2ox3, encoding a GA 2-oxidase, was isolated from spinach. The recombinant protein converted only two C₂₀-GA precursors, GA₁₂ and GA₅₃, to their respective products, GA₁₁₀ and GA₉₇. GA2ox3 competes with GA2ox1 for their common substrate, GA₅₃. In SD, deactivation to GA₉₇ prevails, whereas in LD conversion to GA₂₀ is favored. Transcript levels of SoGA2ox3 were higher in shoot tips than in blades, petioles, and young leaves. Ectopic expression of SoGA2ox3 in the long-day plant *Nicotiana sylvestris* showed a range of dwarf phenotypes, such as reduced germination, short hypocotyl and stem, dark-green leaves, and late flowering, but normal flowers and seed production. The levels of GA₅₃ and GA₁ were 3- to 5-fold lower in transgenic plants than in wild type, whereas the levels of GA₉₇ and GA₁₁₀ increased 3- to 6-fold in transgenic plants. It is concluded that genetic manipulation of plant stature by increasing deactivation of precursors of active GA is more advantageous than increased deactivation of bioactive GA, itself.

Long-day (LD) rosette plants, such as spinach (*Spinacia oleracea*), grow vegetatively and do not produce a stem when grown under short photoperiods (SD). Upon transfer to LD, stems elongate and flowering is initiated. In spinach, LD-induced stem elongation is dependent on GA-regulated processes. Although 136 different GAs (<http://www.plant-hormones.info/gibberellins.htm>) have been identified from natural sources, most of them are precursors or deactivated catabolites of a few biologically active GAs (Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000; Olszewski et al., 2002). The major endogenous GAs of spinach belong to the early-13-hydroxylation pathway (GA₅₃, GA₄₄, GA₁₉, GA₂₀, GA₁, GA₈, and GA₂₉; Fig. 1A; Talon et al., 1991). Of these GAs, only GA₁ is active per se (Zeevaart et al., 1993). A small gene family of 2-oxidases catalyzes deactivation of GA₁ by 2 β -hydroxylation to GA₈. Genes encoding 2-oxidases have been isolated from several species: *Phaseolus coccineus* and *Arabidopsis* (*Arabidopsis thaliana*; Thomas et al., 1999), pea (*Pisum sativum*; Lester et al., 1999; Martin et al., 1999), rice (*Oryza sativa*; Sakamoto et al., 2001), spinach (Lee and Zeevaart, 2002), and poplar (*Populus* spp.; Busov et al., 2003). These GA

2-oxidases introduce a 2 β -hydroxyl group to the bioactive GAs GA₁ and GA₄ and their respective precursors, GA₉ and GA₂₀. The two recombinant GA 2-oxidases (SoGA2ox1 and SoGA2ox2) isolated from spinach 2 β -hydroxylated the C₁₉-GAs GA₁ and GA₂₀ to GA₈ and GA₂₉, respectively. In addition, SoGA2ox1 also converted the C₂₀-GA GA₅₃ to GA₉₇ (Lee and Zeevaart, 2002). In *Arabidopsis*, two GA 2-oxidases that 2 β -hydroxylated GA₁₂ and GA₅₃ were identified by activation tagging. Increased expression of these genes resulted in decreased levels of active GAs and corresponding dwarf phenotypes in *Arabidopsis* and tobacco (*Nicotiana tabacum*; Schomburg et al., 2003).

In spinach, GA₅₃ is at a branch point and can be converted to bioactive GA₁ via the early-13-hydroxylation pathway, or it can be 2 β -hydroxylated to GA₉₇ (Fig. 1A). In addition to GA₉₇, several other 2 β -hydroxylated GAs are found in spinach: GA₉₈ (2 β -hydroxy-GA₄₄), GA₉₉ (2 β -hydroxy-GA₁₉), and GA₁₁₀ (2 β -hydroxy-GA₁₂; Mander et al., 1996; Owen et al., 1998). Gilmour et al. (1986) found that cell-free extracts from spinach leaves are capable of converting [¹⁴C]GA₁₂ to [¹⁴C]2 β -hydroxy-GA₁₂. These results led us to postulate that an early-2 β -hydroxylation pathway operates in spinach and that a 2-oxidase in spinach specifically 2 β -hydroxylates C₂₀-GAs (Fig. 1A).

Here, we report the molecular cloning and characterization of SoGA2ox3 from spinach that 2 β -hydroxylates two C₂₀-GAs, GA₁₂ and GA₅₃. Ectopic expression of SoGA2ox3 in *Nicotiana sylvestris* produced a high level of GA₉₇ with a concomitant decrease in the levels of active GA₁. Transgenic plants overexpressing this 2-oxidase exhibited GA-deficient

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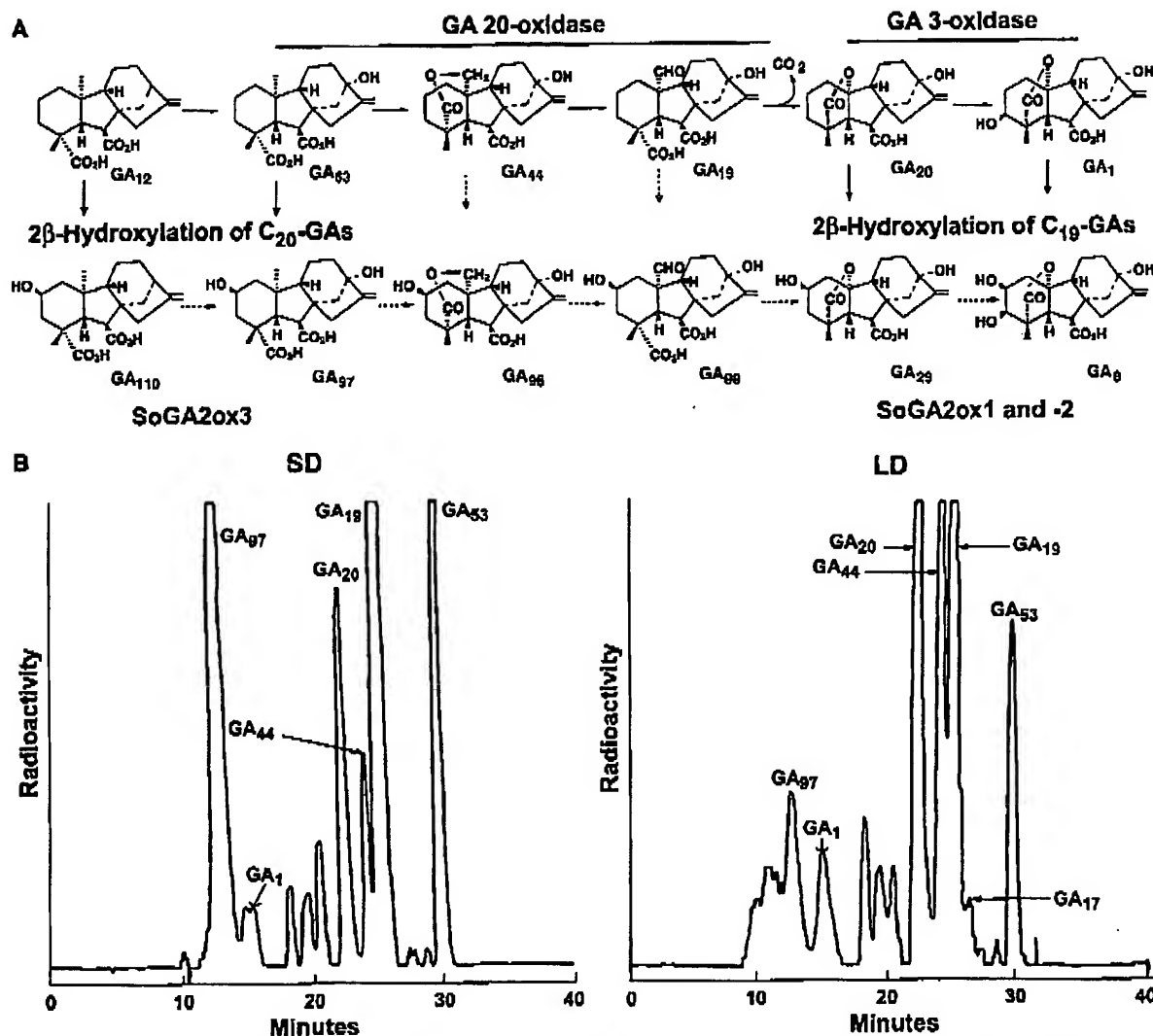


Figure 1. A, The early-13-hydroxylation pathway from GA₁₂ to GA₆ of GA biosynthesis and deactivation in spinach. Each 2β-hydroxylated GA can be derived from the corresponding 13-hydroxylated GA, but only the conversions indicated by solid arrows have been experimentally demonstrated. B, Reverse-phase HPLC profiles of radioactive products after feeding of [¹⁴C]GA₆ to spinach grown in SD or LD. Deactivation of radiolabeled GA₆ by 2β-hydroxylation to GA₉₇ was greater in SD than in LD. Full-scale radioactivity is 10⁴ dpm.

phenotypes, such as delayed germination, short hypocotyl, reduced stature, and late flowering.

RESULTS

Metabolism of [^{14}C]GA₅₃ in Spinach in SD and LD

$[^{14}\text{C}]\text{GA}_{53}$ was fed to spinach plants in SD and LD to determine differences in GA metabolism in plants under various photoperiodic conditions. As shown in Figure 1B, there were striking differences in the metabolism of GA_{53} between plants in SD and LD. First, much more GA_{53} remained unmetabolized in SD

than in LD. Second, much more GA₅₃ was converted to GA₂₀ in LD than in SD. This is expected because GA 20-oxidase activity is much higher in LD than in SD (Lee and Zeevaart, 2002). Third, more radioactive GA₁ was produced in LD than in SD. This is presumably due to the increased level of GA₂₀, the substrate for GA 3-oxidase, which is not up-regulated by LD (Lee and Zeevaart, 2002). Fourth, and most striking, is the massive accumulation of GA₉₇ in plants in SD as compared to those in LD. These data indicate that inactivation of GA₅₃ by 2 β -hydroxylation is predominant in SD, whereas in LD GA₅₃ is preferentially converted to GA₂₀ and further to bioactive GA₁. On the basis of these results, it was of interest to investigate

this GA 2-oxidase and its control at the molecular-genetic level.

Cloning of *SoGA2ox3* cDNA

First-strand cDNA was synthesized with random primers and poly(A⁺) RNA isolated from spinach grown under SD conditions as a template. All combinations of degenerate primers were used in the PCR reactions with first-strand cDNA. The combination of degenerate primers JZ598 and JZ600 (Table I) yielded a 611-bp fragment of a putative GA 2-oxidase (data not shown). The predicted partial amino acid sequence of this reverse transcription (RT)-PCR product shares more identity with the amino acid sequences of AtGA2ox7 and AtGA2ox8 (Schomburg et al., 2003) than with those of the *SoGA2ox1*, *SoGA2ox2*, *AtGA2ox1*, *AtGA2ox2*, and *AtGA2ox3* proteins (Thomas et al., 1999; Lee and Zeevaert, 2002). 5'- and 3'-RACE were performed with sequence-specific primers (JZ616, JZ617, JZ618, and JZ627).

The coding region of *SoGA2ox3* was obtained with primers JZ651 and JZ656 (Table I). The full-length cDNA clone of *SoGA2ox3* (1,242 bp, GenBank accession no. AY935713) has an open reading frame of 1,128 bp, encoding a putative protein of 375 amino acids with a 79-bp 5'-untranslated sequence and a 35-bp 3'-untranslated sequence. The predicted molecular mass of the protein is 43 kD, with a pI of 8.42. Southern blot analysis using the *SoGA2ox3* cDNA as a probe indicated that there is at least one more related gene in the spinach genome (data not shown). Figure 2A shows an alignment of amino acid sequences of GA 2-oxidases from spinach, *Arabidopsis*, and rice. When compared with other 2-oxoglutarate-dependent dioxygenases, the deduced amino acid sequence of *SoGA2ox3* fits best in the family of GA 2-oxidases (Fig. 2B). The predicted amino acid sequence shares 59.3%, 42.4%, 46%, and 45.3% identity with AtGA2ox8, AtGA2ox7, OsGA2ox5, and OsGA2ox6, respectively. However, it shares only 23.1% and 22.1% identity with the *SoGA2ox1* and *SoGA2ox2* proteins (Lee and Zeevaert, 2002). The amino acid sequence of the *SoGA2ox3* protein contains gene-specific motifs (located at positions 143–149, 158–164, and 358–371)

that are different from other GA dioxygenases, and a Thr homopolymer (located at positions 49–58). Three underlined regions show unique amino acid sequences that are highly conserved in *SoGA2ox3*, *AtGA2ox7*, *AtGA2ox8*, *OsGA2ox5*, and *OsGA2ox6* but not in other GA dioxygenases, including *SoGA2ox1* and *SoGA2ox2* (Fig. 2, A and B). The amino acid sequence of *SoGA2ox3* shares several conserved regions with other GA dioxygenases, such as putative 2-oxoglutarate-binding sites (Arg-319 and Ser-321) and iron-binding sites (His-253, Asp-255, and His-309; Thomas et al., 1999). However, the amino acid sequence (L-S-W-S-E-A, positions 157–162) shares only 50% identity with the L-P-W-K-E-T sequence, which is conserved in all GA 2-oxidases and which has been proposed to be involved in binding the substrates GA₁₂ and/or GA₅₃ (Wu et al., 1996; Kang et al., 1999; Sakamoto et al., 2004). *SoGA2ox3* contains one intron of 781 bp (AY935714), which is located at the same position as the second intron of the *AtGA2ox7* and *AtGA2ox8* genes (Schomburg et al., 2003).

The amino acid sequences of all GA 2-oxidases were aligned using the ClustalW 1.8 Multiple Sequence Alignment program, and a phylogenetic tree was generated (Fig. 2B). This analysis divides the GA 2-oxidase family into three different clades. Members of classes I and II catabolize C₁₉-GAs. However, as far as tested, members of class III can only 2 β -hydroxylate C₂₀-GAs (*SoGA2ox3*, *AtGA2ox7*, and *AtGA2ox8*). The two GA 2-oxidases of rice in class III, *OsGA2ox5* and *OsGA2ox6*, have not been characterized, but their amino acid sequences predict that they are able to 2 β -hydroxylate C₂₀-GAs.

Heterologous Expression of *SoGA2ox3* in *Escherichia coli*

To demonstrate that the *SoGA2ox3* cDNA clone encodes a GA 2-oxidase, we heterologously expressed the coding region as a fusion protein of the glutathione S-transferase fusion vector in *E. coli* strain BL21pLysS. The size of the fusion protein is about 69 kD. Soluble protein extracts were used for assays of GA 2-oxidase activity with several radioactive GAs as substrates. The reaction products were separated by reverse-phase HPLC with an on-line radioactivity detector

Table 1. Primers used for amplification of the GA 2-oxidase3 gene from spinach

Single-letter codes for nucleotides were assigned as follows: Y (C/T), N (A/T/C/G), R (A/G), D (A/G/T), and W (A/T).

Purpose	Primer	Primer Sequence (5' to 3')	Orientation
RT-PCR	JZ598	GARTGGGGNTTYYTTCARRT	S
RT-PCR	JZ600	GCYTGRAATAWRTCNCCDATTRT	AS
3'-RACE	JZ617	GCATGATGAGCTGACCTTTATTG	S
3'-RACE	JZ618	AGATACCCACCATGCCCTAAAT	S
5'-RACE	JZ616	TTGAATGGCTCCCTAAACACTT	AS
5'-RACE	JZ627	ACCGTTGACCATAGATTGCTGA	AS
Coding region	JZ651	TCCGGATCCAAATGGCTTACCAAGGTAG	S
Coding region	JZ656	CTGAGAATTCCTACTTCGAAATGACGAAG	AS

Table II. Identification of products formed after incubation of recombinant GA 2-oxidase (SoGA2ox3) from spinach with GA₁₂ or GA₅₃

Substrate	Product	Mass Spectra of Products ^a	
		<i>m/z</i> (% relative abundance)	
17,17- ² H ₂ GA ₁₂	17,17- ² H ₂ GA ₁₁₀	M ⁺ 450 (5), 435 (6), 418 (25), 390 (44), 375 (4), 360 (2), 328 (6), 318 (7), 300 (100), 285 (66), 274 (40), 260 (26), 259 (22), 241 (98), 225 (24), 201 (17), 197 (10), 145 (34)	
17,17- ² H ₂ GA ₅₃	17,17- ² H ₂ GA ₉₇	M ⁺ 538 (35), 523 (9), 506 (6), 479 (9), 448 (3), 389 (10), 373 (5), 329 (11), 299 (2), 239 (44), 210 (65), 209 (100), 195 (11), 179 (16), 147 (3), 119 (14)	

^aAs the methyl ester trimethylsilyl ethers.

to identify the products of GA₁₂ and GA₅₃ catabolism by gas chromatography-mass spectrometry (GC-MS; Table II). Recombinant SoGA2ox3 did not convert [¹⁴C]GA₄₄ (closed lactone ring) and [¹⁴C]GA₁₉ to 2β-hydroxy-[¹⁴C]GA₄₄ (=GA₅₃) and 2β-hydroxy-[¹⁴C]GA₁₉ (=GA₉₇), respectively. Also, SoGA2ox3 was not able to convert C₁₉-GAs, such as GA₂₀ and GA₁. Thus, SoGA2ox3 belongs to the class of 2-oxidases that specifically deactivates the C₂₀-GA precursors GA₁₂ and GA₅₃ (Schomburg et al., 2003), which are also the substrates for GA 20-oxidases.

Effects of Daylength on Gibberellin Levels in Spinach

The GA contents of spinach plants growing in SD or LD were determined by GC-MS-selected ion monitoring (Table III). As reported previously (Wu et al., 1996), transfer of spinach from SD to LD caused an increase in all GAs of the early-13-hydroxylation pathway, except for GA₅₃ in mature leaves and petioles. By contrast, the levels of the 2β-hydroxylated C₂₀-GAs GA₉₇ and GA₁₁₀ showed a decrease after transfer from SD to LD, which corroborates the results obtained with feeding [¹⁴C]GA₅₃ (Fig. 1B). GA₉₇ is by far the most abundant GA in spinach, whereas GA₁₁₀ is present in levels comparable to those of the other GAs. This striking difference in contents of GA₉₇ and GA₁₁₀ raises the question whether SoGA2ox3 has a higher affinity for GA₅₃ than for GA₁₂. To answer this question, we

incubated mixtures of the two substrates at equimolar concentrations with recombinant SoGA2ox3 for 1 h. GA₁₂ and GA₅₃ were equally well catabolized over a range of substrates from 30 to 300 pmol (data not shown), indicating that the much higher content of GA₉₇ than of GA₁₁₀ is probably not due to different affinities of the enzyme for the two substrates.

Expression of SoGA2ox3 in Spinach in SD and LD

Two sizes of transcripts of SoGA2ox3 were observed around 1.3 kb and 0.7 kb in northern-blot analysis (data not shown). The larger size was assumed to be the mature message because it is the same size as the full-length cDNA (see above).

To investigate how expression of the SoGA2ox3 gene is regulated by the photoperiod, we isolated total RNA from spinach plants grown in SD and after 8 LD. Figure 3A shows that the levels of SoGA2ox3 transcripts were relatively high in petioles and shoot tips but low in blades and young leaves in both SD and LD. These expression patterns of SoGA2ox3 are correlated with the levels of GA₉₇ in various organs of spinach (Fig. 3A; Table III).

To determine the time course of changes in SoGA2ox3 expression, spinach plants were harvested at different times after transfer from SD to LD. The levels of SoGA2ox3 transcripts were slightly increased in

Table III. Comparison of GA levels in various organs of spinach in SD and after 8 LD

Spinach plants were grown in SD for 6 weeks, then transferred to LD for 8 d. GA content was determined by GC-MS-selected ion monitoring, using deuterated GAs as internal standards.

Plant Part	GA ₅₃	GA ₁₉	GA ₂₀	GA ₁	GA ₂₉₋₃₁	GA ₉	GA ₁₁₀	GA ₉₇
Mature blades								
SD	4.6 ^a	31.8	2.7	1.7	13.5	1.3	8.5	191.1
8 LD	2.1	23.5	23.6	2.7	36.4	3.2	1.3	73.0
Petioles								
SD	24.6	51.3	1.7	1.6	2.7	2.9	18.9	477.5
8 LD	21.7	145.3	36.7	4.1	42.3	32.3	4.7	446.0
Young leaves								
SD	7.2	54.4	3.0	4.5	10.1	1.3	16.2	286.3
8 LD	31.1	138.4	43.4	19.4	84.7	9.9	2.4	178.7
Shoot tips								
SD	24.7	61.3	2.2	3.8	16.0	3.8	18.3	880.0
8 LD	66.6	173.5	31.4	16.4	66.7	37.1	13.4	696.2

^aValues are in ng g⁻¹ dry weight.

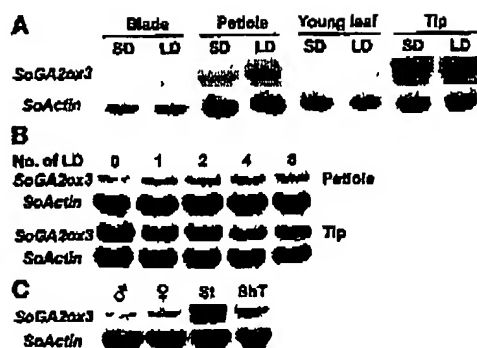


Figure 3. Northern analysis of *SoGA2ox3* in spinach. A, Levels of *SoGA2ox3* transcripts in spinach grown in SD and after 8 LD. B, Levels of *SoGA2ox3* transcripts in petioles and tips with increasing numbers of LD. C, Levels of *SoGA2ox3* transcripts in male (♂) and female (♀) flowers, stems (St), and shoot tips (ShT). Spinach plants were harvested 4 weeks after transfer to LD. The shoot tips included the upper 1 cm of shoots; stems were the next 1 cm of shoots. Total RNA (30 µg) was loaded for each sample. The blots were probed with *SoGA2ox3* or *SoActin*.

blades and young leaves (data not shown) and petioles in a time-dependent manner, but the levels of *SoGA2ox3* transcripts decreased slightly in shoot tips with increasing duration of LD (Fig. 3B).

Expression patterns of *SoGA2ox3* were also investigated in inflorescences. Male and female flowers, stems, and shoot tips were harvested from spinach plants that had been exposed to LD for 4 weeks (Fig. 3C). The *SoGA2ox3* transcripts were more abundant in the upper 2 cm of stems than in shoot tips, and expression was relatively low in male and female flowers.

Ectopic Expression of the *SoGA2ox3* Gene in *N. sylvestris*

To determine whether ectopic expression of the *SoGA2ox3* gene would produce GA_{27} and cause a GA-deficient phenotype in another species, we introduced the 35S::*SoGA2ox3* cDNA construct into *N. sylvestris* by *Agrobacterium tumefaciens*-mediated transformation. Ten homozygous lines expressing *SoGA2ox3* were selected (Fig. 4), which exhibited a profound range of dwarfed phenotypes (data not shown). To ascertain the presence of the *SoGA2ox3* gene in the transgenic plants, we performed genomic PCR amplification with a pair of primers, JZ651 and JZ656 (Table I). The PCR products (1.1 kb) were detected without any other PCR products in all transgenic lines (data not shown). Phenotypes of transgenic lines 6 to 10 were very mild, but transgenic lines 1 to 5 showed severe dwarfed phenotypes (data not shown). The severity of the phenotype was correlated with the accumulation of *SoGA2ox3* transcripts (Fig. 4). The expression patterns of *NsGA20ox1* of *N. sylvestris* were similar to those of *SoGA2ox3* transcripts (Fig. 4), indicating that feedback regulation to maintain GA homeostasis operated in the transgenic plants.

Transgenic line 2 was chosen for further characterization (Fig. 5). In SD, the leaves were slightly shorter and darker green than those of the wild type. The leaves of wild-type plants were smooth, whereas those of transgenic plants were somewhat wrinkled (Fig. 5A). The inset in Figure 5B shows that *SoGA2ox3* was highly expressed in the transgenic plant. The time course of stem elongation (Fig. 5C) shows that the transgenic plants did not start to elongate until the wild-type plants had already reached their final height and were in full bloom. The final height of the transgenic plants was less than half of that of the wild-type plants, and the number of leaves was almost doubled (Table IV). Flower buds in the transgenic plants appeared 30 d later than those of the wild type (Fig. 5C; Table IV). However, the interval between appearance of flower buds and anthesis was the same in wild-type and transgenic plants, indicating that the rate of flower bud development was the same in both types of plants. Fruit and seed production were normal in the transgenic plants. This may indicate that these aspects of plant development require a lower GA_1 level than stem growth. As in leaves (Fig. 4), *SoGA2ox3* was highly expressed in flower buds and mature flowers (Fig. 6). Expression of native *NsGA20ox1* and *NsGA20ox2* in young flower buds was up-regulated as compared to their expressions in the wild type. However, expression of native 20-oxidases in mature flowers was below the limit of detection in northern blots (Fig. 6). Thus, at an early stage, GA 20-oxidase could channel sufficient GA_{53} through the early-13-hydroxylation pathway for normal flower development. It should be kept in mind, however, that flowers are heterotrophic, so that GAs, instead of being synthesized in situ, may be supplied by green tissue.

Effect of Ectopic Expression of *SoGA2ox3* on Gibberellin Content

As expected, GA levels were affected by ectopic expression of *SoGA2ox3* in transgenic plants (Table V). The level of GA_{53} , which is the substrate for both GA 20-oxidase and GA 2-oxidase3, showed a marked decrease in transgenic plants in both SD and LD. The levels of GA_{19} and GA_{20} , the products of GA 20-oxidase, also decreased in transgenic plants in LD with a concomitant 5-fold decrease in bioactive GA_1 . Overexpression of *SoGA2ox3* did not affect the GA_{27} content

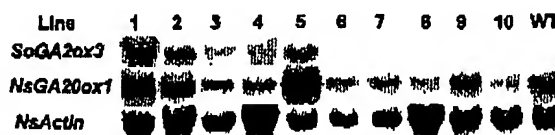


Figure 4. Northern analysis of *SoGA2ox3* and *NsGA20ox1* expression in transgenic *N. sylvestris*. Expression levels of the *SoGA2ox3* transgene parallel those of the *NsGA20ox1* gene. For each sample, 30 µg of total RNA was loaded. The blots were probed with *SoGA2ox3*, *NsGA20ox1*, or *NsActin*.

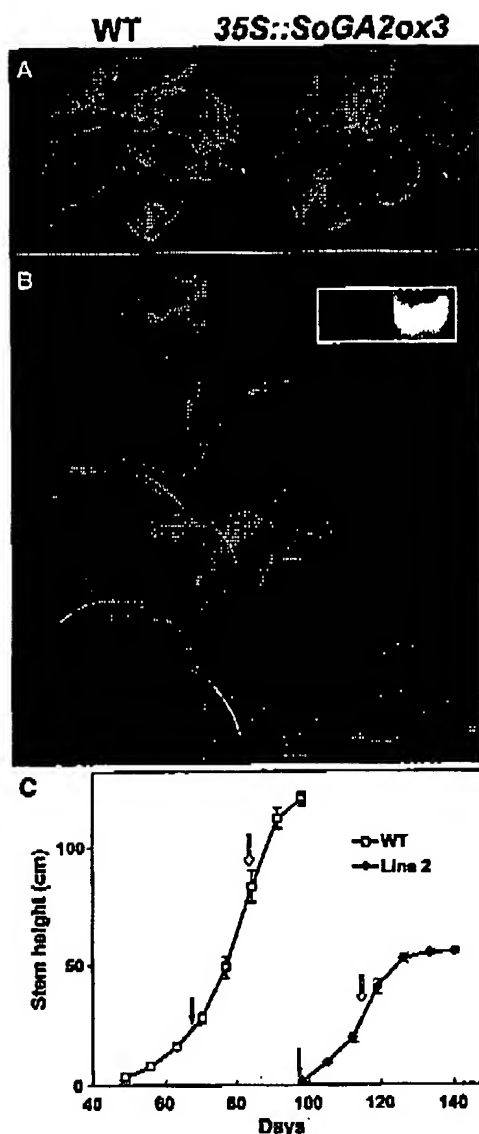


Figure 5. Ectopic expression of *SoGA2ox3* suppresses stem growth in *N. sylvestris*. A, Transgenic plant and wild-type plant (WT) show a similar phenotype in SD. B, WT and transgenic plants grown in LD following germination. Inset in B shows expression of *SoGA2ox3* in the transgenic plant. C, Stem growth of WT and transgenic plants grown in LD following germination. Black arrows indicate the average number of days until appearance of flower buds. White arrows indicate the average number of days until anthesis. $n = 12$.

in SD, but it caused a 3- to 6-fold increase in transgenic plants in LD. As in spinach, GA_{110} was present in much lower amounts than GA_{97} ; its levels also increased in LD. These data indicate that ectopic expression of *SoGA2ox3* increased the levels of both GA_{97} and GA_{110} in transgenic plants and kept the GAs of the early-13-hydroxylation pathway at a low level. Thus, GA 2-oxidase3 in transgenic plants competes effectively with GA 20-oxidase for the common substrates

GA_{12} and GA_{53} , resulting in a reduced GA_1 content and a dwarfed phenotype.

Applied Gibberellin Restores a Normal Phenotype in *35S::SoGA2ox3* Plants

Treatment with GA_1 reversed the phenotype of dwarf transgenic plants to that of wild type. In SD, leaves were elongated and the plants had started to bolt after 25 d. Although grown in SD, the GA_1 -treated plants had the appearance of wild-type plants exposed to LD (Fig. 7A). In LD, the transgenic plants treated with GA_1 showed essentially the same phenotype as wild-type plants (Fig. 7B). These results show that GA_1 treatment effectively increased leaf and stem growth of transgenic plants both in SD and LD, indicating that application of GA_1 could fully compensate for the decreased endogenous GA_1 content of transgenic plants.

Effect of Ectopic Expression of *SoGA2ox3* on Germination

Because severely GA-deficient mutants do not germinate in the absence of exogenous GAs (Koorneef et al., 1983), we tested whether overexpression of *SoGA2ox3* affects germination. As shown in Figure 8, a high percentage of wild-type seeds had germinated after 4 d, whereas germination of transgenic seeds did not begin until day 5. Treatment with GA_3 accelerated germination and also caused a higher percentage of seeds to germinate than was observed for seeds without GA_3 treatment (Fig. 8). Thus, although overexpression of *SoGA2ox3* decreases the content of bioactive GA, transgenic seeds are still able to germinate, albeit with some delay.

Effect of Ectopic Expression of *SoGA2ox3* on Hypocotyl and Root Growth

A role of GA in the regulation of root growth has been established by the use of GA-deficient mutants (Yaxley et al., 2001; Fu and Harberd, 2003). It was of interest, therefore, to investigate whether root growth is affected in seedlings with a low GA_1 content due to overexpression of *SoGA2ox3*. As shown in Figure 9, ectopic expression of the *SoGA2ox3* gene markedly affected both hypocotyl and root growth. The hypocotyls of wild-type seedlings were elongated in darkness, but those of transgenic seedlings remained very

Table IV. Characterization of transgenic plants overexpressing *SoGA2ox3* in *N. sylvestris* (Ns)

Observed Trait	Wild-Type Ns	<i>35S::SoGA2ox3</i>
Days to flower buds	67.3 \pm 0.92*	97.8 \pm 0.86
Days to anthesis	83.5 \pm 0.97	114.5 \pm 0.96
Number of leaves to inflorescence	19.0 \pm 0.35	35.8 \pm 0.35
Final plant height	122.6 \pm 2.8	56.1 \pm 1.01

*SE; $n = 12$.

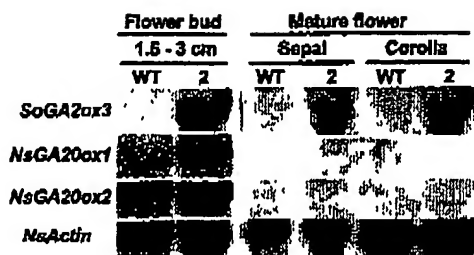


Figure 6. Northern analysis of *SoGA2ox3*, *NsGA2ox1* (GenBank accession no. AF494087), and *NsGA2ox2* (accession no. AF494088) in young flower buds and mature flowers of transgenic *N. sylvestris*. For each sample, 30 μ g of total RNA was loaded. The blots were probed with *SoGA2ox3*, *NsGA2ox1*, *NsGA2ox2*, or *NsActin*.

short (Fig. 9A). Treatment with 1 mg L⁻¹ GA₃ completely restored hypocotyl length of transgenic seedlings to that of wild type (Fig. 9B). Differences in root growth between wild-type and transgenic seedlings were most pronounced under continual light (Fig. 9C). Treatment with GA₃ could only partially restore root growth in transgenic seedlings, but hypocotyl growth was promoted to the same extent in both types of seedlings (Fig. 9D).

DISCUSSION

In this study, we isolated a full-length cDNA clone encoding a GA 2-oxidase from spinach. Heterologous expression in *E. coli* showed that the product of *SoGA2ox3* catalyzes 2 β -hydroxylation of the C₂₀-GAs GA₁₂ and GA₅₃ to GA₁₀ and GA₉₇, respectively. The same activities have previously been found for AtGA2ox7 and AtGA2ox8 (Schomburg et al., 2003). The recombinant proteins from other GA 2-oxidases characterized so far 2 β -hydroxylate C₁₉-GAs. The only exceptions reported are for PcGA2ox1 and AtGA2ox2, which 2 β -hydroxylated GA₁₅, a C₂₀-GA (Thomas et al., 1999); and OsGA2ox1, which converted GA₄₄ to GA₉₈ (Sakamoto et al., 2001). Spinach produces GA₉₈ and GA₉₉, but recombinant *SoGA2ox3* did not metabolize their possible precursors, GA₄₄ and GA₁₉. In transgenic *N. sylvestris* overexpressing *SoGA2ox3*, there was an

increase in GA₉₇ (Table V), but no GA₉₈ and GA₉₉ could be detected in these plants (J.A.D. Zeevaart, unpublished data). Considering these combined results, it is unlikely that *SoGA2ox3* is involved in the synthesis of GA₉₈ and GA₉₉. Another possibility is that GA 2-oxidase converts GA₉₇ to GA₉₈ and GA₉₉, as indicated in the hypothetical pathway in Figure 1A. But GA₉₇ was not converted by recombinant *SoGA2ox3* (J.A.D. Zeevaart, unpublished data). Perhaps recombinant *SoGA2ox1* can 2 β -hydroxylate GA₄₄ and GA₁₉, as it was able to convert GA₅₃ to GA₉₇ (Lee and Zeevaart, 2002). Finally, the possibility remains that there are additional GA 2-oxidases that catalyze the formation of GA₉₈ and GA₉₉.

GA₉₇ is much more abundant than GA₁₁₀ in spinach (Table III), and the same is true for *N. sylvestris* in both wild-type and transgenic plants (Table V). Given the results of the experiment in which the two precursors GA₁₂ and GA₅₃ were mixed, it is unlikely that GA 2-oxidase3 has a higher affinity for GA₅₃ than for GA₁₂. The cellular location of the GA 13-oxidase that converts GA₁₂ to GA₅₃ is not known, but there may be compartmentation of the substrates in such a manner that GA₅₃ is more readily available to GA 2-oxidase3 than is GA₁₂. Further metabolism of GA₉₇ is not known, so that its very high level may not reflect a high rate of synthesis but rather accumulation of a stable inactive end product. A relatively low rate of synthesis of GA₉₇ is also indicated by the low levels of *SoGA2ox3* transcripts in spinach (Fig. 3). In addition, antiserum raised against recombinant *SoGA2ox3* protein did not detect any *SoGA2ox3* protein from spinach or transgenic *N. sylvestris* in western blots, indicating that the level of *SoGA2ox3* protein in vivo is very low (D.J. Lee, unpublished data).

GA₅₃ is at a branch point in GA metabolism in spinach (Fig. 1A). It can be converted to bioactive GA₁ via the early-13-hydroxylation pathway, or it can be deactivated by 2 β -hydroxylation to GA₉₇. The latter step appears to prevail in SD, whereas in LD most GA₅₃ will be channeled through the early-13-hydroxylation pathway. As shown in Figure 3, the levels of *SoGA2ox3* transcripts undergo little change when spinach plants are shifted from SD to LD. Neverthe-

Table V. GA content of *N. sylvestris* (Ns) and transgenic plants overexpressing *SoGA2ox3* grown under various photoperiodic conditions

Plants were grown in SD for 80 d and then transferred to LD for the number of days indicated prior to harvest. GA content was determined by GC-MS-selected ion monitoring, using deuterated GAs as internal standards. nd, Not detectable.

Sample	Photoperiodic Treatment	GA ₅₃	GA ₁₉	GA ₂₀	GA ₁	GA ₉₇	GA ₁₁₀
Ns	SD	0.7 ^a	3.8	1.2	0.9	11.7	nd
Ns/35S::SoGA2ox3	SD	0.1	2.1	0.3	0.8	11.6	0.5
Ns	14 LD	13.2	32.4	16.7	4.9	16.9	nd
Ns/35S::SoGA2ox3	14 LD	4.4	4.7	0.8	0.9	40.3	7.2
Ns	20 LD	32.3	54.3	27.0	5.2	14.1	1.5
Ns/35S::SoGA2ox3	20 LD	6.3	7.5	1.8	1.0	85.3	9.1
Ns/35S::SoGA2ox3	30 LD	9.7	9.4	2.3	1.1	94.9	10.2

^aValues are in ng g⁻¹ dry weight.



Figure 7. Reversal of dwarf phenotype of transgenic plants by GA treatment. Plants were sprayed twice weekly with a solution of 10 mg L⁻¹ GA₁. GA treatment of transgenic plants in SD (A) and in LD (B). Photographs were taken 25 d after first GA application.

less, the levels of GA₅₇ decreased, presumably because expression of GA 20-oxidase was strongly up-regulated in LD (Lee and Zeevaart, 2002). Thus, the fate of GA₅₃ will be determined by the relative activities of the two enzymes GA 2-oxidase3 and GA 20-oxidase. In LD, 20-oxidase activity is high, so that GA₅₃ is preferentially converted to GA₂₀. By contrast, in SD the activity of 20-oxidase is low, and more GA₅₃ will be deactivated via 2 β -hydroxylation to GA₉₇. Whereas SoGA2ox1 and SoGA2ox2 can directly regulate the level of active GA₁, SoGA2ox3 can only indirectly regulate the level of active GA₁ by reducing the amount of precursor that is channeled through the early-13-hydroxylation path-

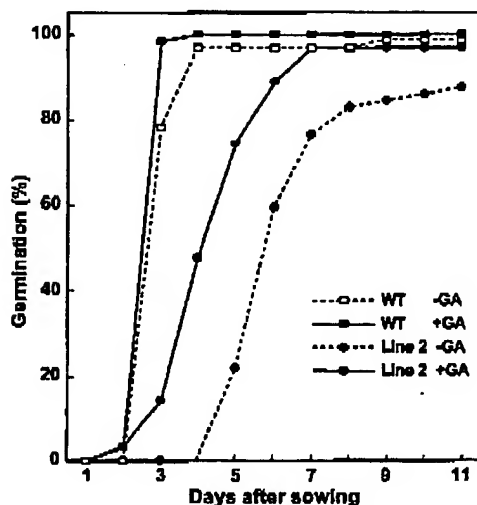


Figure 8. Effect of overexpression of SoGA2ox3 on germination in *N. sylvestris*. Seeds of wild-type (WT) or transgenic plants were spread on agar plates containing half-strength Hoagland solution. Treatment with GA partially restored germination of transgenic seeds. $n = 60$.

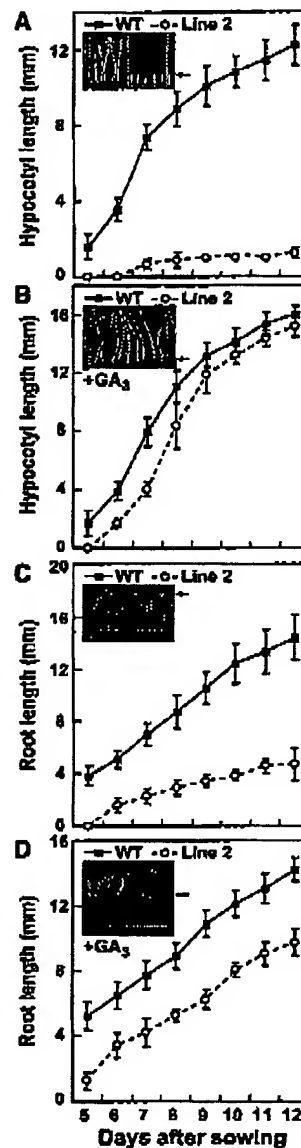


Figure 9. Effect of overexpression of SoGA2ox3 on hypocotyl and root growth in *N. sylvestris* seedlings. A, Hypocotyl length of seedlings grown in darkness. B, Treatment with GA restored to normal the hypocotyl growth of transgenic seedlings in darkness. C, Root growth of transgenic seedlings grown under continuous light. D, Treatment with GA partially restored the root growth of transgenic seedlings under continuous light. Arrows indicate transition from root to hypocotyl. Photographs taken 15 d after sowing. $n = 15$.

way. Thus, 2 β -hydroxylation of GA precursors of bioactive GA₁ adds an additional step at which the levels of active GAs can be regulated.

Overexpression of SoGA2ox3 in *N. sylvestris* had a profound effect on growth and development from germination to flowering (Figs. 5, 7, 8, and 9). Analysis of the endogenous GAs showed a reduced GA₁ level in transgenic plants and an increase in GA₉₇ and GA₁₁₀.

Wild-type *N. sylvestris* also produced GA₂₇, although not at the high levels found in spinach. The reduction in active GA₁ resulted in reduced germination, shorter radicles and hypocotyls of seedlings, reduced stature, and later flowering. The GA-deficient phenotype of older plants could be readily overcome by applied GA₁ (Fig. 7).

Application of GA to the LD rosette plant *N. sylvestris* in SD induces stem elongation and, ultimately, flower formation (Lang, 1989). As shown in Table V, the level of GA₁ increased 5- to 6-fold when plants were transferred from SD to LD. This increase indicates that a high level of bioactive GA is a prerequisite for stem elongation and subsequent flowering. Conversely, a reduced level of GA₁ in transgenic plants delayed stem elongation and flower formation (Fig. 5; Table IV). This retardation in flowering is most vividly expressed by the number of leaves formed prior to the inflorescence in transgenic plants compared to wild-type plants (Table IV).

Semidwarfism is a desirable trait in agriculture (Busov et al., 2003; Hedden, 2003). Overexpression of GA 2-oxidase is an easy way to reduce GA levels in transgenic plants, but ectopic expression of *SoGA2ox1* and *SoGA2ox2* in *N. sylvestris* caused severely dwarfed plants that produced few seeds (D.J. Lee, unpublished results) because active GA was probably deactivated as soon as it was produced. Singh et al. (2002) also reported that ectopic expression of class II GA 2-oxidases (*PsGA2ox2*) caused defective pollen tube growth and seed abortion. Overexpression of *AtGA2ox1* in tobacco reduced the number of capsules 40% to 70% (Biemelt et al., 2004). In rice, ectopic expression of *OsGA2ox1* inhibited not only stem elongation but also development of reproductive organs. In a more direct approach, *OsGA2ox1* was expressed under the control of the promoter of *OsGA3ox2*, which is specifically expressed in the shoot apex. This caused a semidwarf phenotype with normal flowering and grain development (Sakamoto et al., 2003). When *SoGA2ox3* was overexpressed, GA precursors were deactivated, but GA 20-oxidase could still convert a small amount of the precursor to GA₁ (Table V), which could act without being quickly deactivated. This finding demonstrates that we can manipulate plant stature by overexpressing a gene that deactivates precursors of GA₁ rather than active GA₁ itself. The result is plants with reduced GA₁ levels, short stature, and delayed flowering but that otherwise retain desired traits, such as normal flowering and seed production.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Spinach (*Spinacia oleracea*, Savoy Hybrid 612) plants were grown in a SD growth chamber for approximately 6 or 7 weeks as described earlier and then transferred to a LD growth chamber for experimental use (Lee and Zeevaart, 2002). *Nicotiana sylvestris* Spegaz. et Comes was grown under the same conditions. Plants used for GA extraction were grown in SD for approximately 3 months before being exposed to LD. All plants were watered with half-

strength Hoagland solution daily. SD consisted of 8 h of light from fluorescent tubes and incandescent bulbs of approximately 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 23°C, followed by 16 h darkness at 20°C. In the case of LD, the 8-h main light period was followed by 16 h of weak light from incandescent bulbs of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Feeding of [¹⁴C]GA₃₃ to Spinach in SD and LD

[¹⁴C]GA₃₃ (specific activity 127.5 mCi/mmol) was dissolved in a small amount of acetone, to which water containing 0.05% (v/v) Tween 20 was added to give a 75% (v/v) aqueous solution. This solution was injected into petioles and midribs of spinach leaves by means of a 100- μL Hamilton syringe. Each plant received 0.3×10^6 dpm of [¹⁴C]GA₃₃ (=0.36 μg GA₃₃). Spinach plants were either kept in SD or had been exposed to 8 LD. The plants, 4 per treatment, were harvested after 2 d, frozen in liquid N₂, and lyophilized. The dried material was extracted with methanol, and an acidic fraction was prepared from each sample (Zeevaart et al., 1993; Schomburg et al., 2003). The acidic fractions were analyzed by reverse-phase HPLC with on-line radioactivity detection. Radioactive fractions were collected, the derivatized materials were identified, and their specific activities determined by GC-MS (Zeevaart et al., 1993).

RT-PCR

RT-PCR was used for the cloning of GA 2-oxidase. First-strand cDNA from 1 μg poly(A⁺) RNA was synthesized by SuperScript II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA), using oligo(dT) primers at 42°C for 1 h. The cDNA product was then used in PCR reactions containing *Taq* polymerase and several pairs of degenerate primers (Table 1). For GA 2-oxidase, the degenerate primers were synthesized based on conserved regions of *AtGA2ox7* and *AtGA2ox8* (Schomburg et al., 2003). These primers were used in all combinations in PCR reactions with first-strand cDNA as a template. The PCR amplifications were performed with a DNA thermal cycler (RTC-200; MJ Research, Waltham, MA) in a 50- μL reaction mixture containing 1 \times PCR buffer, 1.5 mM MgCl₂, 200 μM dNTP, 2.5 pmol of each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen). The reaction mixtures were heated to 94°C for 2 min and then subjected to 30 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 45 s. A final extension was performed at 72°C for 10 min. A 5- μL volume of the PCR reaction products was analyzed by 1.2% agarose gel electrophoresis, purified with a Wizard PCR purification system (Promega, Madison, WI), and then cloned into the pGEM-T Easy vector (Promega).

RACE and Cloning

5'- and 3'-RACE of spinach GA 2-oxidase3 cDNA were performed using the SMART RACE cDNA amplification kit from CLONTECH (Palo Alto, CA). Poly(A⁺) RNA was isolated from spinach using the PolyATtract mRNA Isolation System IV (Promega) according to the manufacturer's instructions. Following RT with primers supplied by CLONTECH, the first-strand cDNA was used directly in 5'- and 3'-RACE PCR reactions. Primary PCR amplification reactions were achieved using a high-fidelity enzyme (*Pfu* Turbo polymerase; Stratagene, La Jolla, CA) and gene-specific primers J2616 and J2627 or J2617 and J2618 for *GA2ox3* to generate the 5'- or 3'-cDNA RACE fragments, respectively. The PCR reaction consisted of the first denaturation for 3 min at 94°C, a series of 30 cycles (1 min at 94°C, 1 min at 54°C or 55°C, 1 min at 72°C), and a final extension for 5 min at 72°C using a thermal cycler (MJ Research). A 5- μL aliquot of the RT-PCR and RACE reaction solution was analyzed by 1.2% agarose gel electrophoresis. PCR products were purified and cloned into the pCR-Script Carni SK(+) cloning vector from Stratagene. These constructs were sequenced.

The National Center for Biotechnology Information Blast program was used to search genes homologous with the *SoGA2ox3* gene. Analysis of the DNA sequences was carried out using the DNASTAR program (DNASTAR, Madison, WI). Multiple sequence alignment of the amino acid sequences was performed using BCM Search Launcher (<http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>) and printed with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic tree was created by means of the ClustalW program (<http://www.ebi.ac.uk/clustalw/>).

Northern-Blot Analysis

Leaf blades, petioles, young leaves, shoot tips, and other organs were harvested, frozen immediately in liquid N₂, and stored at -80°C (Lee and

Zeevaert, 2002). Northern blots were prepared by electrophoresis of 30 μ g of total RNA in the presence of formaldehyde (Sambrook et al., 1989), and RNA was transferred to nitrocellulose. Full-length cDNAs were labeled with [32 P]dCTP by the Random Primers DNA labeling system (Invitrogen) and used to probe northern and Southern blots. Hybridization was carried out at 42°C using a 50% formamide system (Sambrook et al., 1989). Membranes were washed twice in $2 \times$ SSC at room temperature for 10 min and then twice in $0.2 \times$ SSC with 0.1% SDS at 55°C for 10 min. At low stringency, membranes were washed once in $2 \times$ SSC at room temperature for 10 min and then in $2 \times$ SSC at 55°C for 10 min. Each blot was first probed with *SoGA2ox3* cDNA, stripped, and reprobed with *SoActin*. Each experiment was repeated at least three times with similar results.

Expression of Recombinant GA 2-Oxidase3 Protein

The coding region of GA 2-oxidase3 was produced by PCR with the following primer set designed from the KACB product: JZ651 and JZ656 for the mature *GA2ox3* cDNA (*Bam*HI and *Eco*RI sites inserted to facilitate cloning). The resulting PCR fragments were cloned into the pGEM-T Easy cloning vector, digested with *Bam*HI/*Eco*RI, and subcloned into the corresponding restriction sites of the pGEX-SK-2 vector (Amersham Biosciences, Piscataway, NJ). This full-length cDNA clone (pGEXGA2ox3) was transformed into *Escherichia coli* strain BL21pLysS. Fifty mL of freshly cultured cells were added to 1 L of Luria-Bertani medium with 100 mg L⁻¹ ampicillin and incubated at 37°C with vigorous shaking. When the optical density at 600 nm reached 0.6, isopropyl- β -D-thiogalactopyranoside was added to give a final concentration of 3 mM, and the culture was incubated for another 2 h. The cells were harvested, suspended, and incubated in lysis buffer (100 mM Tris-HCl, pH 7.5, and 10 mg L⁻¹ lysozyme) at room temperature for 10 min. Crude extracts were briefly sonicated by using a Sonifier Cell Disruptor 200 (Branson Ultrasonics, Danbury, CT), carried out for 10 cycles (10 \times 10 s) on ice. The lysates were submerged in liquid N₂ for 2 min and then thawed in an ice bath for 15 min (Johnson and Hocht, 1994). The lysates were centrifuged at 13,000 rpm for 30 min, and the supernatant was stored at -80°C until needed for enzyme assays.

Enzyme Assays and Product Identification

Enzyme assays with recombinant GA 2-oxidase3 were performed with approximately 30,000 dpm of [14 C]-labeled GAs. The assays and methods for product identification have been described (Lee and Zeevaert, 2002; Schomburg et al., 2003).

Gibberellin Extraction and Quantification

The procedures for extraction, purification, and quantification of endogenous GAs with deuterated GAs as internal standards were the same as described (Talon and Zeevaert, 1990; Zeevaert et al., 1993). All GA measurements were repeated at least twice with similar results. The four prominent ions monitored for GA₇ were mass-to-charge ratio (*m/z*) 477/479 and 536/538, and for GA₁₀ *m/z* 298/300 and 388/390. Deuterated GA₇ and GA₁₀ were prepared by means of incubating 50 μ g of 17,17- 2 H₂GA₃ and 17,17- 3 H₂GA₁₂, respectively, with 2.5 mL of recombinant *SoGA2ox3* protein. The GC-MS conditions were as described by Schomburg et al. (2003).

Construction of 35S::SoGA2ox3 Plasmid and Transformation

The full-length *SoGA2ox3* cDNA was introduced into the pGEM-T Easy vector, and then reintroduced into the *Bam*HI and *Sac*I sites of the pBI121 vector after removing the *GUS* gene. This vector, pBI*SoGA2ox3*, was introduced into *Agrobacterium tumefaciens* strain LBA4404, for transformation of *N. sylvestris* according to Horsch et al. (1988). Leaf discs of *N. sylvestris* were incubated with *A. tumefaciens* for 10 min and then transferred to Murashige and Skoog medium containing 1 mg L⁻¹ 6-benzylaminopurine and 0.1 mg L⁻¹ 1-naphthaleneacetic acid (NAA) in darkness. After coculture for 3 d, leaf discs were transferred to selection medium (Murashige and Skoog, 1 mg L⁻¹ 6-benzylaminopurine, 0.1 mg L⁻¹ NAA, 100 mg L⁻¹ kanamycin, and 500 mg L⁻¹ carbenicillin). When shoots appeared, individual shoots were cut and transferred to root inducing medium (Murashige and Skoog, 0.1 mg L⁻¹ NAA, 100 mg L⁻¹ kanamycin, and 500 mg L⁻¹ carbenicillin). After rooting, plantlets

(T₀) were planted in soil and grown in a greenhouse. Independent transgenic lines were selected on half-strength Hoagland medium containing 50 mg L⁻¹ kanamycin. The T₁ generations were self-pollinated to get T₂ generations. Each line showing a 3:1 segregation (resistant:sensitive) was selected to obtain homozygous T₃ plants; these homozygous lines were used in further experiments.

Analysis of Transgenic Plants by PCR

Isolation of genomic DNA from transgenic plants was performed as described (Lee and Kim, 1998). The PCR amplification reaction was for 25 cycles of 30 s at 94°C, 60 s at 50°C, and 60 s at 72°C with two primers (JZ651 and JZ656; Table I) after an initial denaturation step for 2 min at 94°C. The 5 μ L of reaction products was analyzed by 1.2% agarose gel electrophoresis.

Germination Assays

Sixty seeds were sown in petri dishes containing half-strength Hoagland medium in the presence or absence of 1 mg L⁻¹ GA₃ and incubated in a growth room at 23°C under continual light from fluorescent lamps (50–80 μ mol m⁻² s⁻¹). Germination was defined as radicle emergence and was scored daily.

Hypocotyl and Root Growth

Growth of hypocotyls and roots was determined by sowing seeds on half-strength Hoagland medium containing 0.8% phytoagar with or without 1 mg L⁻¹ GA₃. The petri dishes were vertically oriented so that the seedlings could grow vertically along the agar surface. Wild-type and transgenic lines were sown in the same petri dish at 23°C. Seedlings were kept in darkness to determine hypocotyl length, or kept under continuous light (same source as used for germination assays) to determine root growth. Both hypocotyl length and root length were measured until 12 d after sowing.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY935713 (*SoGA2ox3* cDNA) and AY935714 (intron of *SoGA2ox3*).

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